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Identification of the Enzymes Responsible for the Detoxification of Furfural and 5-Hydroxymethyl-2-furalaldehyde in *Zymomonas mobilis*

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Introduction

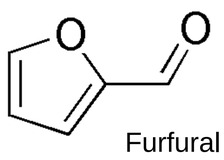
Lignocellulosic Feedstock
Crop residues, forestry products, energy crops

Heat and Mild Acid Pretreatment
Produces monomeric sugars ready for fermentation

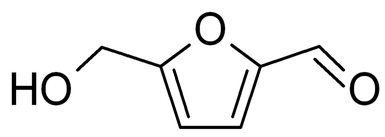
Fermentation
Sugars are converted to Ethanol by Zymomonas moblilis (*Z. moblis*)

•Heat and mild acid is used to treat lignocellulosic material in order to depolymerize sugars, making them readily available for fermentation by microorganisms.

▪Mild acid treatments increase the bioavailablility of fermentable sugars, but also produce an array of byproducts that inhibit the growth and metabolism of *Z. moblis*. One group of highly potent inhibitors are called furalaldehydes. Two furalaldehydes in particular, furfural and 5-hydroxymethyl-2-furalaldehyde (HMF) are strong inhibitors with known deleterious biological effects.



Furfural



Hydroxymethyl-2-furaladehyde (HMF)

▪One approach to circumvent furaldehyde toxicity is to enhance the ability of the fermenting organism to process and neutralize furaldehydes. In order to pursue this approach it is essential that we first understand how furaldehydes are metabolized. Previous work on this approach suggests that furfural is reduced to furfuryl alcohol, and that this compound is much less toxic to *Z. mobilis.* The *Z. mobilis* genome encodes two alcohol dehydrogenases (adh), adhI and adhII, but only AdhI reduces furfural. It is unknown if *Z. mobilis* has additional furfural reduction pathways. The goal of this study is to identify which protein(s) are responsible for furfural reduction and determine their relative contribution to overall furfural activity.

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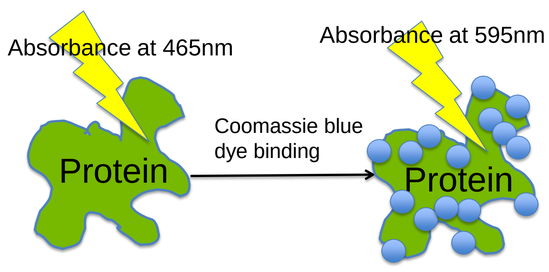
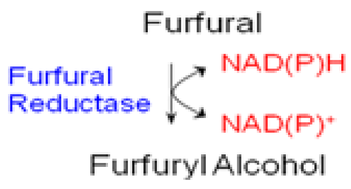
Method

Furfural reduction activity and protein concentrations were monitored at each step of fractionation in order to track protein separation efficacy. Furfural activity was measured by tracking the conversion of NAD(P)H to NAD(P)$^+$ by measuring the decrease in absorbance at 340nm. Protein concentrations were monitored by measuring the absorbance shift from 465nm to 595nm upon protein binding to Coomassie Blue dye.

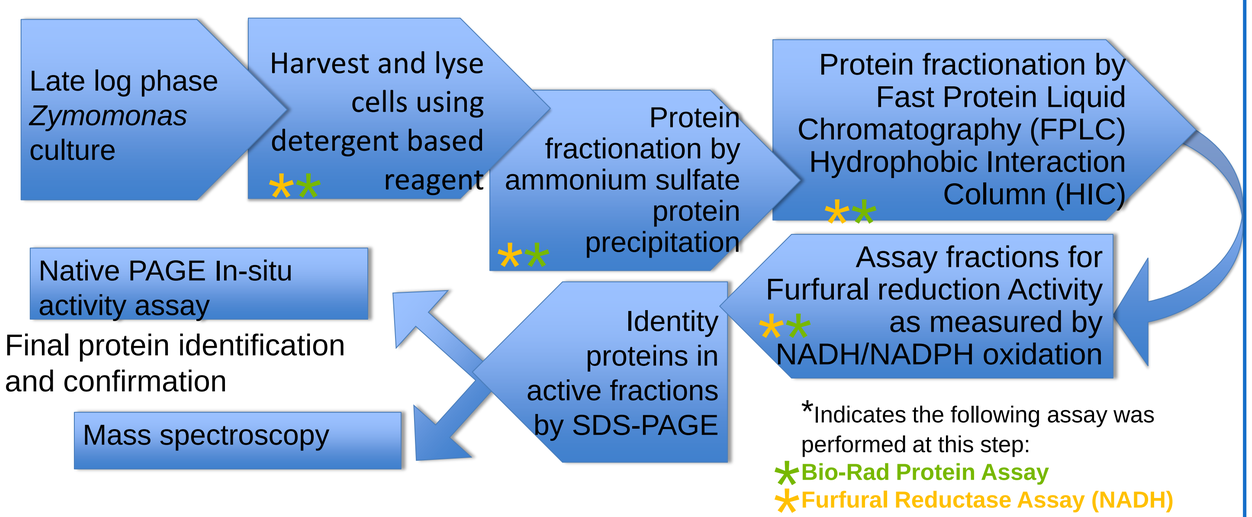
Bio-Rad Protein Assay



Furfural Reductase Assay



Protein Fractionation Protocol



*Indicates the following assay was performed at this step:
*Bio-Rad Protein Assay
*Furfural Reductase Assay (NADH)

Results: Method Optimization

Before purification the following variables were optimized to produce as much activity and protein as possible:

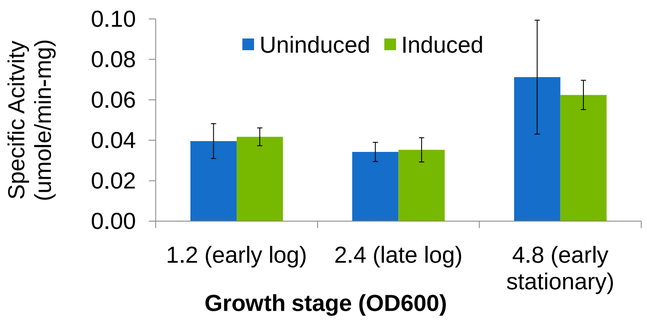


Figure 1. Induction activity was not inducible with furfural, and activity is greatest in early stationary phase

Initial Investigations:
•NADH works as a cofactor for furfural reduction, but NADPH does not
•Ammonium sulfate precipitates furfural reduction proteins at 75% (v/v), but not at 60% (v/v).
•Approximately 2M ammonium sulfate is necessary for protein to bind to the Resource ISO HIC column (GE)

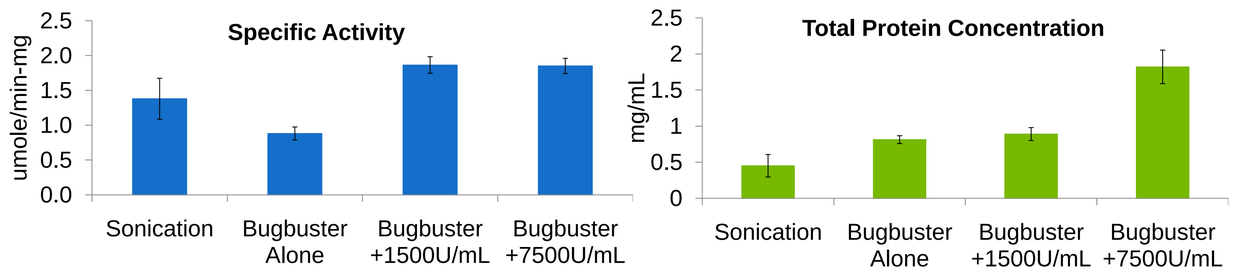
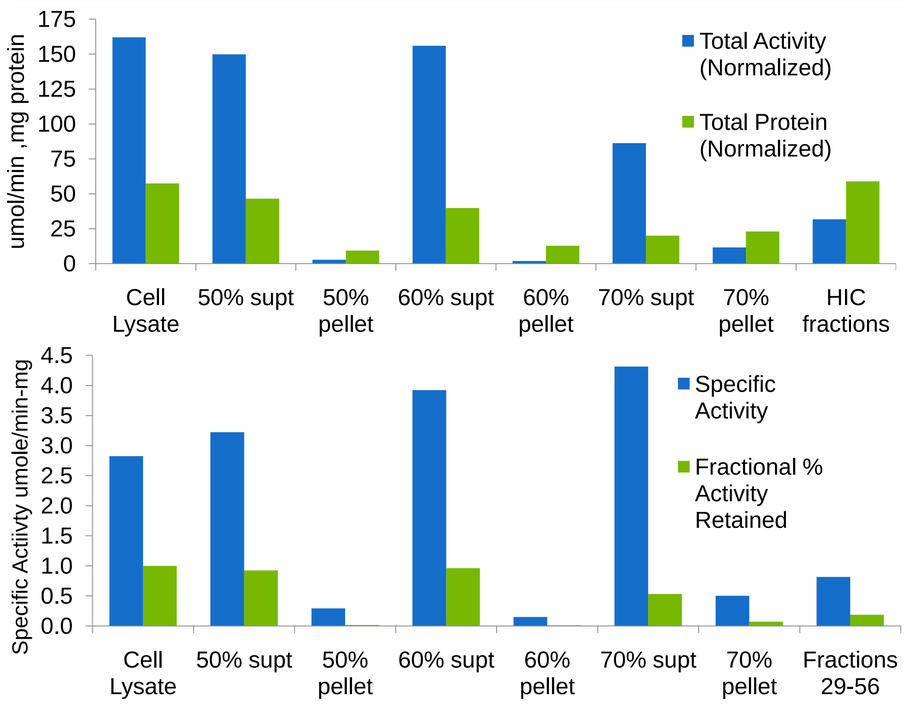


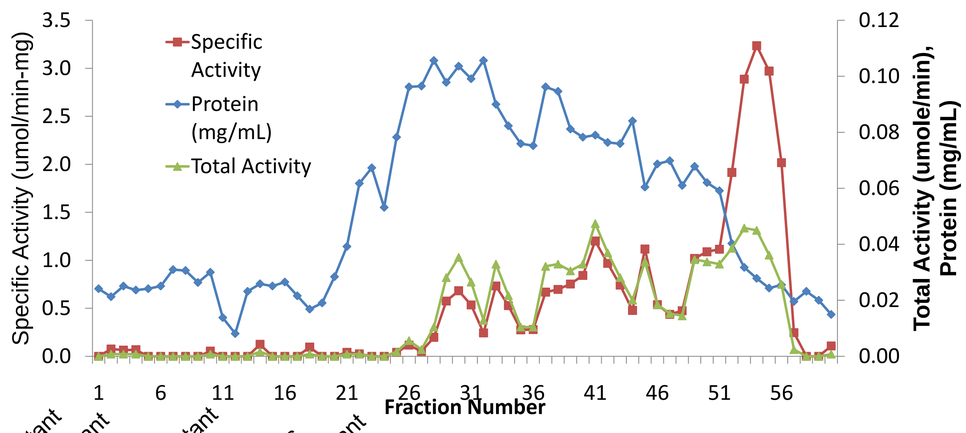
Figure 2. Optimization of lysis conditions. Use of Bugbuster HT® detergent based lysis reagent with added lysozyme (7500 units) improved specific activity yield along with total protein yield.

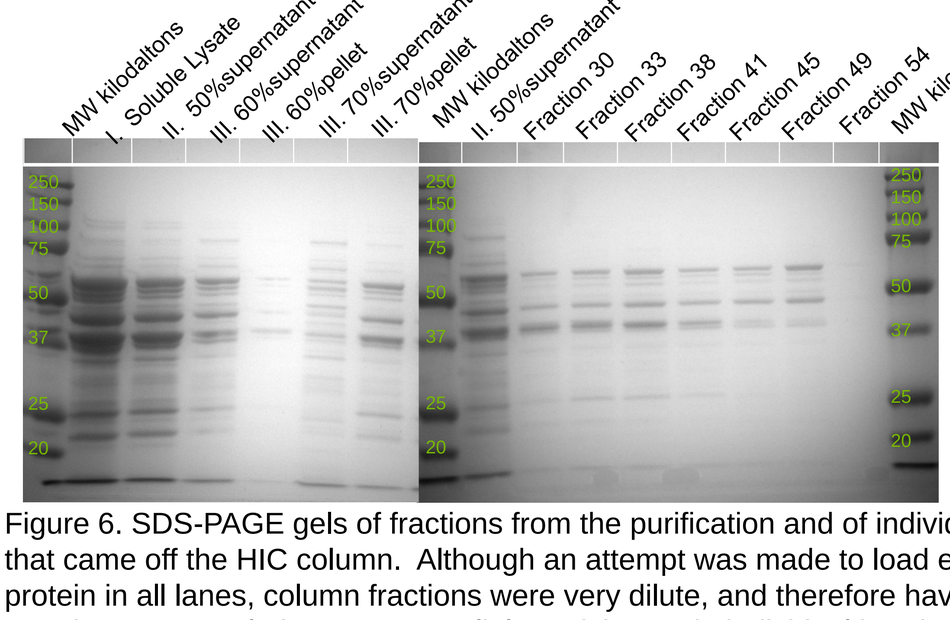
Results: Purification and Separation



Figures 3 and 4. Protein (mg) with furfural reductase activity (umole/min) started to precipitate around 70% ammonium sulfate. Therefore, the soluble fraction from the 60% ammonium sulfate precipitation is a good starting material for purification. Specific activity decreased to 1/3 the initial, raw lysate, value for FPLC fractions with furfural reduction activity (29-56).

Figure 5. Furfural reduction activity and protein concentration by fraction. Furfural reduction activity was spread among many peaks and may represent more than one protein with activity towards furfural.





10% Sodium dodecysulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples were run for one hour at 200V Stained with Coomassie Blue (Biorad)

Figure 6. SDS-PAGE gels of fractions from the purification and of individual fractions that came off the HIC column. Although an attempt was made to load equal total protein in all lanes, column fractions were very dilute, and therefore have less total protein. In general, there was very little enrichment in individual bands, however some fractionation did take place

Conclusions

We successfully designed and optimized assays for measuring protein concentrations in high ammonium sulfate conditions, furfural reduction activity, and the lysis and extraction of active *Z. mobilis* proteins. We additionally developed and optimized methods for cell fractionation including ammonium sulfate precipitation, and FPLC fractionation based on hydrophobicity. Cell preparation and ammonium sulfate precipitation protocols improved specific activity approximately 30%, and lay the groundwork for more FPLC column optimization. We also found furfural reduction activity to be NADH, not NADPH, dependent and not inducible with furfural. SDS-PAGE showed that there was very little enrichment of individual protein bands, which correlates with our data showing broad furfural activity that distributed across many protein peaks. Future work will focus on manipulating column conditions in order to more effectively separate *Z. mobilis* proteins and recover total furfural reductase activity. Once separation is enhanced, it will be possible to correlate peaks of high activity with protein peaks, and ultimately identify proteins with furfural reductase activity. FPLC variables to be tested include using different FPLC columns, such as ion exchange, or even a stronger HIC, loading larger quantities of total protein, using higher concentrations of protein, and also using larger columns and lysate volumes.

Acknowledgements

I would like to thank the members of the FTLB 230 Lab for their help with equipment, reagents, and techniques. I would also like to especially thank Min Zhang and Mary Ann Franden for their assistance in planning and implementing experiments. Thanks to Steve Decker for training and use of the FPLC, and Phil Pienkos for helpful discussions. Finally, Linda Lung and Jennifer Coughlin for their many contributions.

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